DECLARATION

I, Sabine Frieda Katharina Town, declare that I am a citizen of the Federal Republic of Germany, residing at Waldstraße 45, 82386 Oberhausen, Federal Republic of Germany, that I am fluent in German and English, that I am a competent translator from German into English and that the attached is a true and accurate translation made by me into the English language of the Patent Application filed in the German Patent and Trademark Office on the 26th March 1998, file Number 198 13 317.0.

I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issuing thereon.

I hereby subscribe my name to the foregoing declaration, this thirteenth day of July 2005.

Signature of translator: ______________________

Sabine F.K. Town

FEDERAL REPUBLIC OF GERMANY

Certificate

The Boehringer Mannheim GmbH in Mannheim/Germany has on the 26th March 1998 filed an application for a patent with the German Patent and Trademark Office entitled

"Improved method of primer extension preamplification PCR".

The attached documents are a correct and accurate reproduction of the original documents of this patent application.

The application received the provisional International Patent Classification symbols C 12 Q and C 12 P in the German Patent and Trademark Office.

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Improved method of primer extension preamplification PCR

The invention concerns an improved method of "whole gene amplification" (WGA) which is suitable for performing a DNA analysis starting with just one or only a few cells. The improvement of the method is essentially achieved by using a mixture of two DNA polymerases to amplify the DNA of which at least one polymerase has 3' – 5' exonuclease activity.

WGA methods are especially important in the field of differential tumour diagnostics. The aim of differential tumour diagnostics at the molecular level is to analyse nucleic acid samples from single cells or cell populations that contain no non-malignant cells (Emmert-Buck et al., 1996; Böhm and Wielang, 1997). Cell sorting methods (Abeln et al., 1994, Barret et al., 1996), microdissection methods (Shibata et al., 1992, Shibata et al., 1993, Emmert-Buck et al., 1994, Noguchi et al., 1994, Zhuang et al., 1995, Böhm et al., 1997) and the method of laser micro-dissection (Schütze and Clement-Sengewald, 1994) are being used increasingly to obtain appropriate samples.

However, particularly sensitive methods of nucleic acid amplification are a prerequisite for the molecular analysis of single cells or populations of a low number of cells. According to the prior art methods of "whole gene amplification" (WGA) are particularly suitable for this. These are methods which consist of two consecutive amplification reactions. The first amplification reaction is carried out using randomized primers and the second amplification is carried out using specific primers. WGA can for example be used to diagnose hereditary diseases as part of preimplantation diagnostics of biopsied blastomer cells (Kristianson et al., 1994,

Snabes et al., 1994, van der Veyer et al., 1995) or in prenatal diagnostic testing of nucleiated erythrocytes in maternal blood (Sekizawa et al., 1996).

According to the prior art WGA is usually used to analyse microsatellites in tumour biopsies to detect microsatellite instability or the loss of heterozygosity. According to the prior art the analysed sample must, however, contain a sufficient number of cells to rule out a quantitative disproportionate amplification of individual alleles due to random preparation artefacts (Zhang et al., 1992, Barret et al., 1995, Cheung and Nelson, 1996, Faulkner and Leigh, 1998). For example a batch of about 1000 cells was examined for a microsatellite analysis of FACS-sorted aneuploid oesophagus tumour cells (Barret et al., 1995).

In contrast to conventional in-situ hybridization (Van Ommen et al., 1995) or to conventional specific PCR (Becker et al., 1996), WGA methods enable multiple analyses of the same sample. A distinction is made between two different methods: In "degenerate oligonucleotide primer PCR" (DOP-PCR) amplification primers are used with defined sequences at the 5' and 3' ends and with a randomized hexamer region in the middle of the primer (Telenius et al., 1992). Starting with less stringent conditions during the first 5 thermal cycles, the subsequent 35 thermal cycles are carried out under more stringent conditions at a higher annealing temperature so that during these cycles only completely complementary primers are still able to bind to the DNA to be amplified. These methods are used for example as a first step before an in-situ hybridization with flow-sorted chromosomes (Blennow et al., 1992, Telenius et al., 1992; Kallionemie et al., 1994) or for comparative genomic hybridization CGH (Du-Manoir et al., 1993; Schlegel et al., 1995).

An alternative WGA principle is the so-called "primer extension preamplification" (PEP-PCR, Zhang et al., 1992). In contrast to DOP-PCR completely randomized 15mer amplification primers are used. During 50 consecutive thermal cycles a denaturation is firstly carried out at 92°C which is followed by a hybridization under less stringent temperature conditions at 37°C which is successively increased to 55°C at a rate of about 0.1° C/sec. The polymerase extension reaction takes place at this temperature for a further 4 minutes.

However, all methods known in the prior art (von Eggeling and Spielvogel, 1995) have the disadvantage of inadequate sensitivity because a relatively large number of cells has to be used to obtain an amplification product with a high degree of probability. In addition the sensitivity of the assay is reduced even more as the length of the fragment to be amplified increases. Hence the methods known in the prior art had only been used to amplify relatively small fragments with a length of up to 580 base pairs (Snabes et al., 1994).

Another major disadvantage of the PEP-PCR known in the prior art is especially that it has hitherto not been possible to reliably carry out a convincing DNA mutation analysis due to the inherent error rate of the Taq polymerase used. The error rate is due to the fact that the use of Taq polymerase during the amplification results in AT/GC transitions in the amplification product (Keohvong + Thily, 1989). Moreover, deletion mutations may be formed when Taq polymerase is used if the DNA to be amplified is capable of forming secondary structures (Carriello et al., 1991). However, the risk of obtaining amplification artefacts is especially high with WGA because more than 80 amplification cycles are usually carried out during the two to three amplification reactions.

It was also known in the prior art that DNA polymerases with 3' – 5' exonuclease activity can be used to avoid sequence artefacts during nucleic acid amplification (Flaman et al., 1994; Casas + Kirkpontrick, 1996). However, the use of polymerases without 3' – 5' exonuclease activity for WGA leads to a further reduction of the sensitivity of the method since such polymerases have a substantially lower processivity than Taq DNA polymerases. Consequently the products generated during the preamplification using randomized primers are not long enough to serve as a template for the subsequent specific PCR reaction if the fragment to be amplified exceeds a certain size.

Hence the technical object to be solved at the time of the invention was to develop a method which, starting with the smallest possible number of cells, can be used to amplify specific nucleic acid fragments in a high quality i.e. without sequence artefacts and subsequently analyse them. The quality of the amplification products

should make it possible to carry out reliable mutation analyses, sequence analyses and unequivocally interpretable microsatellite analyses. This object is achieved by an improved method of primer extension preamplification (PEP-PCR, Zhang et al., 1992).

Hence the invention concerns a method for amplifying nucleic acid fragments from a sample which comprises two or three thermocyclic amplification reactions in which completely randomized primers are used during the first amplification reaction and specific primers are used in the second amplification reaction, characterized in that a mixture of at least two DNA polymerases is used to amplify the DNA of which at least one polymerase has 3' – 5' exonuclease activity. This property is also referred to as proof-reading activity in the case of polymerases (Flaman et al., 1994).

An amplification reaction comprises about 20 to 60 thermal cycles where preferably at least 40 thermal cycles and particularly preferably at least 50 thermal cycles are carried out during the first amplification reaction. Preferably least 30 thermal cycles and particularly preferably at least 40 thermal cycles are carried out for the second amplification reaction.

Each thermal cycle consists of a denaturing phase, an annealing phase and at least one elongation phase. Denaturation into single strands preferably takes place at temperatures between 90°C and 96°C. The annealing phase to hybridize the primers to the target nucleic acid preferably takes place at temperatures between 30°C and 50°C. The annealing phase particularly preferably takes place between 35°C and 45°C. During the first amplification reaction the annealing phase particularly preferably takes place at approximately 37°C. The elongation phase is carried out at temperatures between 50°C and 75°C. In a preferred embodiment the elongation phase of the first amplification reaction takes place at temperatures between 50°C and 60°C, a temperature of approximately 55°C being particularly preferred.

It is advantageous for carrying out the claimed invention when there is a slow transition from the annealing phase to the elongation phase, this transition being

carried out at a rate of less than 0.5°C/second. The temperature transition is particularly advantageously carried out at a rate of 0.1°C/second.

For carrying out the method according to the invention it has also proven to be advantageous to carry out the elongation using two or more elongation steps in the majority of the cycles during the first amplification reaction, with one elongation being firstly carried out at a lower temperature and then the elongation being continued at a higher temperature. In this manner populations of particularly long amplicons are generated during the first amplification reaction. In this embodiment the first amplification reaction preferably takes place at about 55°C and the second amplification reaction at about 65°C to 72°C, a temperature of about 60°C having proven to be optimal.

The primers used in the first amplification reaction are completely randomized i.e. a population of single-stranded oligonucleotides is used in which each individual nucleotide can consist of one of the four nucleotide building blocks A, T, G or C at each individual position. These primers preferably have a length of 10 – 20 nucleotides. Primers having a length of about 15 nucleotides are particularly preferred. The specific primers used in the second amplification reaction are characterized in that they have a sequence which is identical to a sequence of the target nucleic acid or of its complementary sequence over a region of at least 10 nucleotides. The specific primers used in a potential third amplification reaction to carry out a "nested PCR" are selected according to the same criteria as the primers of the second amplification reaction in which case the sequences of the primers used that are identical to the target nucleic acid or its complement must be a component of the sequence amplified in the second amplification reaction.

The inventive mixture of DNA polymerases preferably contains a thermostable DNA polymerase without 3' – 5' exonuclease activity such as Taq DNA polymerase and another thermostable DNA polymerase with 3' – 5' exonuclease activity such as Pwo DNA polymerase from Pyrokokkus woesii (Boehringer Mannheim, Catalogue No. 1644947). Other DNA polymerases without 3' – 5' exonuclease activity can also be used as a component of the polymerase mixture.

One of the embodiments of the claimed method is a method for DNA amplification. In this case it has proven to be advantageous for the sensitivity of detecting certain sequences to carry out a cell lysis of the material to be analysed with the aid of an enzymatic protease digestion in order to isolate the sample DNA. Proteinase K can for example be used for this.

In another embodiment of the method according to the invention RNA is firstly isolated from the body material to be analysed, the starting point being 1 cell, less than 10 cells or less than 100 cells. A corresponding cDNA is produced using a reverse transcriptase reaction which is subsequently used as a starting material for the primer extension preamplification according to the invention. The cDNA is preferably obtained by reverse transcription of poly-A RNA.

The claimed methods are suitable for analysing samples of body material which consist of one or only a few cells. Hence the claimed method is also particularly suitable for analysing nucleic acids from tissue sections. Such tissue sections can be obtained from frozen material as well as from tissue that is fixed by formalin or embedded in paraffin. An appropriate protease digestion is particularly advantageous for these embodiments.

The use of polymerase mixtures for primer extension preamplification PCR according to the invention results in a surprisingly high sensitivity of DNA detection that cannot be achieved using the methods known in the prior art. Hence a subject matter of the invention is a method for amplifying nucleic acid fragments comprising two or three thermocyclic amplification reactions, wherein completely randomized primers are used during the first amplification reaction and specific primers are used in the second amplification reaction, where in addition the sample contains a quantity of nucleic acid corresponding to an equivalent of no more than 100 cells which is characterized in that the amplificates are formed with a probability of more than 90 %. In particular the invention concerns methods in which amplificates are formed with a probability of more than 90 % when using an equivalent of no more than 5 – 10 cells. In a specific embodiment amplificates are

formed with a probability of more than 50 % when using a cell equivalent of one cell.

The method according to the invention is suitable for amplifying nucleic acid fragments having a length between 100 and 1000 base pairs. The method is particularly suitable for amplifying nucleic acid fragments having a length of 150 and 550 base pairs.

In summary the method according to the invention enables the amplification of specific DNA fragments from nucleic acid samples that have been obtained from only one or a few cells while ruling out or at least minimizing the generation of amplification artefacts. Hence a subject matter of the invention is also the use of a DNA amplified according to the invention for mutation analysis. The mutation analysis can be carried out in a special embodiment by analysing the nucleic acid fragment amplified according to the invention with the aid of a sequencing reaction.

A further subject matter of the claimed invention is the use of the DNA amplified according to the invention to analyse microsatellites and in particular to analyse nucleic acid samples that have been obtained from frozen sections or sections of formalin-fixed or paraffin-embedded tissue. Cell equivalents of 5 – 20 cells are preferably used in this embodiment because a uniform amplification of alleles of the same gene locus can no longer be ensured with even lower amounts of cells. The analysis of microsatellites according to the invention can be used to diagnose microsatellite instability and also to diagnose the loss of heterozygosity (Boehm and Wieland, 1997).

Moreover, the likelihood of obtaining amplification products of both alleles is greater than 90 % when using a cell equivalent of at least 10 cells.

Brief description of the figures

Figure 1: Amplification of a 536 bp fragment of the β-globin gene from FACS-sorted SW480 cells. Ten assays each were carried out with 1, 5, 10 and 100 cells.
Row A: Use of an Expand polymerase mixture after lysis with proteinase K
Row B: Use of Taq polymerase after lysis with proteinase K
Row C: Previously-known PEP-PCR after alkaline lysis
Row D: DOP-PCR

Figure 2: Microdissected material from frozen tissue sections of a bladder carcinoma biopsy in aliquots of about 50, 100, 200, 500 or 1000 cells. Amplification of a 536 bp fragment after lysis with proteinase K (A) or using Taq polymerase after alkaline lysis (B).

Figure 3: Microsatellite analysis of the locus D2S123 of cell line SW480 with 10 samples each containing 1, 5, 10 or 100 cells.

Figure 4: Microsatellite analysis of the locus D2S123 from microdissected frozen tissue sections of a bladder carcinoma biopsy on aliquots of about 10, 25, 50 or more than 1000 cells each.

Upper gel row A: Preamplification according to the invention after enzymatic lysis with an Expand polymerase mixture.

Lower gel row B: Preamplification with Taq polymerase after alkaline lysis.

Figure 5: Microsatellite analysis of the locus D2S123 from microdissected sections of a colon carcinoma biopsy that were fixed in formalin and embedded in paraffin on aliquots of about 30, 50, 100 or 250 cells each.

Upper gel row A: Preamplification according to the invention after enzymatic lysis with an Expand polymerase mixture.

Lower gel row B: Preamplification with Taq polymerase after alkaline lysis.

Figure 6: Mutation analyses of the P53 gene using DNA from breast carcinoma biopsies that were preamplified according to the invention.

Figure 7: Detection of Ki-ras mutations in CK18-stained, disseminated tumour cells from bone marrow using RFLP. The DNA was applied in each case in parallel undigested and digested with MvaI.

Lanes 1, 2: CK18-positive single cells from a patient with pancreatic carcinoma.

Lanes 4, 5: CK18-positive single cells from a patient with colon carcinoma.

Lanes 3, 6, 7: A few CK18-positive cells from a patient with colon carcinoma.

Lanes 8-11: About 100 unstained, haematopoetic stem cells each as negative controls.

Figure 8: Amplification of a 408 bp fragment of the β2-microglobulin gene using cDNA isolated from single cells.

Upper gel: Lane 1: Size marker, HindIII-digested lambda DNA; lanes 2 to 13: preamplification PCR according to the invention from 12 different single cells; lane 14: positive control using cDNA from 5000 cells preamplified according to the invention; lane 15: positive control with 1 μg cDNA amplified according to the invention; lane 16: positive control with 1 μg non-preamplified cDNA; lane 17: negative control without reverse transcriptase during the reverse transcription; lane 18: negative control without using cDNA during preamplification.

Lower gel: Lane 1: Size marker, HindIII-digested lambda DNA; lanes 2 to 13: 1/10 aliquots of non-preamplified cDNA from various single cells; lane 14: positive control with 1 μg non-preamplified cDNA.

Example 1:

Sensitivity of the primer extension preamplification according to the invention

In order to compare the method according to the invention with methods known from the prior art, cells from the SW480 tumour cell line (ATCC) were separated using flow cytometry in equivalents comprising 1, 5, 10 or 100 cells. For this purpose the cells were resuspended in PBS buffer containing 0.1 μg/ml fluorescein diacetate and incubated in it for 5 minutes at room temperature. During this the lipophilic, non-fluorescent fluorescein diacetate diffuses through the cell membrane and is subsequently hydrolyzed intracellularly by non-specific esterases thus resulting in negatively charged fluorescein that can no longer diffuse (Endl et al., 1996, Dive et al., 1990, Ross et al., 1989). The fluorescence measurements were carried out using an FAC Starplus cell sorter (Becton-Dickinson). Fluorescence was excited using a water-cooled argon ion laser at a wavelength of 488 nm. The emission was detected in the FL1 channel using a 525 nm band pass filter with a band width of 30 nm. The sorting was adjusted using 20 fluorescein-labelled microbeads (Polysciences, Carboxy YG 4.5, Warrington). The processes were carried out three times and the beads were subsequently counted under a fluorescence microscope. Sorting was considered to be correct if all three sorting processes produced a sorting result of 20 microbeads. Cell analysis was carried out at a rate of about 200 cells per second. Cell sorting was carried out at a drop feed rate of 25,000 Hz directly into Eppendorf vessels that already contained the lysis buffer used. 40 aliquots each of 1, 5, 10 or 100 cells were prepared in this manner.

Cell lysis was carried out according to the invention for 12 hours at 48°C in 10 μl High Fidelity buffer (50 mM Tris-HCl, 22 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, pH 8.9) which additionally contained 4 mg/ml proteinase K and 5 vol % Tween 20 (Merck) followed by inactivating the enzyme for 15 minutes at 94°C. Lysis was performed in parallel batches according to the prior art (Zhang et al. 1992) in 5 μl, 200 mM KOH, 50 mM dithiothreitol for 10 minutes at 65°C, which was followed by neutralizing with 5 μl 900 mM TrisHCl pH 8.3, 300 mM KCl.

Subsequently preamplification according to the invention was carried out for 10 samples each using completely randomized 15mer primers (16 μM) with 5 units of a mixture of Taq polymerase (Boehringer Mannheim) and Pwo polymerase (Boehringer Mannheim) in a ratio of 10:1 under standard PCR buffer conditions (50 mM Tris-HCl, 22 mM $(NH_4)_2 SO_4$, 2.5 mM $Mgll_2$, pH 8.9 additionally containing 1 mg/ml gelatine) in a total volume of 60 μl with the following 50 thermal cycles.

1: 92°C 1' 30"
2: 92°C 40"
3: 37°C 2'
4: ramp: 0.1°C/sec to 55°C
5: 55°C 4'
6: 68°C 30"
7: go to step 2, 49 times
8: 68°C 15'

In control experiments 5 units Taq polymerase (Life Technologies) was used according to the prior art instead of the polymerase mixture according to the invention using samples obtained either by alkaline lysis or by enzymatic lysis. In another control experiment a DOP-PCR was carried out after enzymatic proteinase K digestion using the DOP-PCR Master Kit (Boehringer Mannheim) according to the manufacturer's instructions.

After adding 0.5 μM of each of the primers of sequence ID NO. 1 and 2, a tenth of an aliquot (6 μl) of a 536 bp fragment of the β-globin gene was specifically amplified in the presence of 0.2 mM dNTP and 0.5 μl of the same polymerase in each case that was used during the first amplification round, under PCR standard buffer conditions and using the following thermal cycles:

94°C	3 min	
94°C	1 min	
60°C	1 min	50 x
72°C	1 min	
72°C	10 min	

The amplification products were then analysed on an agarose gel stained with 2 % ethidium bromide. As shown in fig. 1, preamplification according to the invention is clearly superior to the PEP-PCR and DOP-PCR methods in terms of sensitivity of the amplification of a 536 bp β-globin fragment. In the single cell assays, a specific amplification product was obtained in more than 50 % of the cells investigated when the preamplification was carried out using the polymerase mixture according to the invention after enzymatic lysis whereas none of the other methods generated any amplification product.

In assays with 5 (or more) cells, the use of the polymerase mixture after enzymatic lysis yielded an amplification product in every tested sample. The use of Taq polymerase instead of the polymerase mixture resulted in amplification product in 70 % of the 5-cell assays. In contrast amplification products were obtained only in a minority of the tested cell populations using DOP-PCR or the preamplification method known in the prior art i.e. using Taq polymerase after alkaline lysis.

Hence the use of a polymerase mixture for preamplification according to the invention that comprises polymerase with proof-reading activity (3' – 5' exonuclease) and a polymerase without proof-reading activity is advantageous in the analysis of the smallest cell populations all the way down to single cells because its use is very likely to result in a specific amplification product. This significantly increases the sensitivity of such assays. The experiment also demonstrates that performing enzymatic cell lysis instead of alkaline lysis is advantageous for the corresponding assays.

Example 2:

Preamplification of cell material obtained from frozen sections

Frozen sections of 5 µm thickness were prepared from a bladder tumour biopsy and stained with methylene blue (Romeis, 1989). They were microdissected into areas containing about 50, 100, 200, 500 or 1000 cells. Microdissection was performed manually using a micromanipulator (Leitz; serial No. 980629) and an inverted microscope (Leitz, Labovert FS) enlarged 400 times. Subsequently a 536 bp β-globin fragment was preamplified/amplified as described in example 1. As shown in figure 2 a saturated amplification took place in all aliquots analysed provided the preamplification was carried out according to the invention i.e. using an Expand polymerase mixture after lysis with proteinase K. In contrast saturated amplification products were obtained using Taq polymerase after alkaline lysis only if aliquots of at least about 200 microdissected cells were used.

Example 3:

Sensitivity of microsatellite analysis of a tumour cell line

Cells of the cell line SW480 (ATCC) were separated into 10 aliquots each of 1, 5, 10 or 100 cells according to the method described in example 1. Preamplification with an Expand polymerase mixture was carried out according to the invention as described in example 1 after enzymatic lysis with proteinase K.

After the preamplification 1/30 aliquots were used to analyse the microsatellite locus D2S123. 0.3 µm primer of Seq Id. No. 3 and 4 was used for the amplification under standard PCR conditions (Dietmaier et al., 1997). The PCR amplifications were carried out as follows in a volume of 20 µl:

denaturation: 94°C 1 minute
annealing: 60°C 1 minute
elongation: 72°C 1 minute

After being repeated 50 times, the last elongation step was carried out at 72°C for 8 minutes.

3 µl sample buffer (96 % formamide 1 % xylene cyanol ff, 1 % bromophenol blue, 10 mMol EDTA, pH 8.0) was added to 3 µl of the PCR product, it was denatured for 4 minutes at 94°C and separated by gel electrophoresis on a 6.7 % polyacrylamide / 50 % urea gel for 1 hour at 1.500 volts and 50°C in a sequencing gel chamber (Biorad).

As shown in figure 3, the cells of the microsatellite-stable cell line SW480 exhibited a uniform allele type and it was possible to reliably detect both alleles when at least 10 cells were used. In a 5-cell assay both alleles could be detected with a probability of about 80 %, whereas in a single-cell assay it was only possible to detect single alleles at a low success rate.

Example 4

<u>Microsatellite analysis of material obtained from frozen sections</u>

In another experiment a series of microdissociated cells of a bladder tumour biopsy were separated similarly to example 2 in cell equivalents of about 10, 25, 50 and 1000 cells in each case and either alkaline-lysed or lysed with proteinase K. The lysates were subsequently used for microsatellite analysis of the D2S123 locus similarly to example 3. As shown in figure 4 a cell equivalent of 10 cells is already sufficient to reliably obtain allele-specific amplification products when carrying out a preamplification PCR according to the invention i.e. with enzymatic lysis using proteinase K and using an enzyme mixture of Taq polymerase and Pwo polymerase. Both alleles are represented uniformly and generate an unequivocal band pattern that can be evaluated. In contrast, the method of alkaline lysis and subsequent preamplification using Taq polymerase according to the prior art resulted in diffuse band patterns in the analysis of about 10 cell equivalents that could not be unequivocally evaluated.

Example 5

Microsatellite analysis of material from paraffin-embedded tissue

Colon carcinoma tissue was stained with hematoxilin/eosin (Romers, 1989, p. 213), fixed in PBS-buffered formalin (formalin: 3.7 % formaldehyde in PBS) for 14 h, embedded in paraffin and subsequently dissected into 5 µm sections and placed on microscope slides. To destain, the microscope slides were washed for 2*15 min in xylene, 2*10 min in 99.9 % ethanol, 2*10 min in 96 % ethanol and 2*10 min in 70 % ethanol. After microdissection into about 30, 50, 100 and 250 cells, a microsatellite analysis was performed as described in example 4. As shown in figure 5, analysable allele-specific amplification products with uniform representation of both alleles were obtained in the analysis of a cell equivalent of 30 cells by performing a preamplification PCR according to the invention, i.e. using enzymatic lysis with proteinase K and using an enzyme mixture of Taq polymerase and Pwo polymerase. In contrast, the method of alkaline lysis followed by preamplification using Taq polymerase according to the prior art led to diffuse band patterns that often represented only single alleles.

Example 6

P53-mutation analysis

In order to determine the error rate of the improved PEP-PCR method with subsequent gene-specific PCR amplification, genomic regions of the P53 gene (exon 7 and exon 8) from 8 different breast carcinomas were analysed. So-called touch preparations (Kovach et al., 1991) were prepared for this. Similarly to example 2 cell clusters of about 50-60 cells were microdissected and then enzymatically lysed and preamplified according to the invention. Afterwards a P53-specific PCR was carried out. Non-preamplified DNA was amplified under the same conditions as a control reaction. The specific amplification was carried out under standard conditions in a reaction volume of 50 µl (200 nM dNTPs, 1.25 U Expand polymerase (Boehringer Mannheim), 1.5 mM (exon 7) or 2 mM (exon 8) $MgCl_2$ and 0.4 µM of each of the amplification primers using the following thermal cycles:

1 x	94°C	2 min
35 x	94°C	1 min
	50°C	2 min
	72°C	3 min
1 x	72°C	10 min

If a nested PCR was necessary, 2 µl of the first PCR reaction was used. The following primers were used in the various amplification reactions:

Seq. ID. No. 5	exon 7 first round up	5' AAAGGCCTCCCCTGCT 3'
Seq. ID. No. 6	exon 7 first round up	5' GAGCAGTAAGGAGATT 3'
Seq. ID. No. 7	exon 7 sec. round up	5' CTCCCCTGCTTGCCA 3'
Seq. ID. No. 8	exon 7 sec. round down	5' GATGGGTAGTAGTATG 3'
Seq. ID. No. 9	exon 8 first round up	5' GACAGGTAGACCTGAT 3'
Seq. ID. No. 10	exon 8 first round down	5' TCTGAGGCATAACTGC 3'
Seq. ID. No. 11	exon 8 sec. round up	5' AGACCTGATTTCCTTAC 3'
Seq. ID. No. 12	exon 8 sec. round down	5 TAACTGCACCCTTGGTC 3'

The amplification products were sequenced by the cycle sequencing method using the PRISM ready dye terminator sequencing kit and AmpliTaqFS polymerase (Applied Biosystems). For this the amplified DNA fragments were firstly precipitated with polyethylene glycol. 3.2 pmol of a sequencing primer (Seq. ID. No. 5-12) and 8.0 µl premix-containing buffer, dye-labelled ddNTPs, dNTPs and ampli-Taq FS/pyrophosphatase were added to 50 – 150 ng of the precipitate. After denaturing at 96°C for 2 min, the reactions were incubated as follows over 25 cycles:

96°C/15 sec.
50°C/15 sec.
60°C/4 min.

Subsequently the samples were precipitated with ethanol, dried and resuspended in 2 µl sample buffer (5:1 deionized formamide, 0.05 mol EDTA, pH 8), heated for 2

minutes to 92°C and subsequently loaded onto an Applied-Biosystem 373 sequencing apparatus.

A comparison of the sequence analyses of DNA preamplified according to the invention with DNA of the same patients which was used directly for sequencing resulted in no difference in any of the investigated patients. The position and type of identified mutations are shown in figure 6 as an example. Figure 6 shows a heterozygotic 8-base pair deletion in exon 7 (6a), a homozygotic C/G-G/C transversion (6b), a heterozygotic A/T-/T/A transversion (6c) and a heterozygotic A/T-C/G transversion in exon 8 (6d) after primer extension preamplification according to the invention. No further mutations were found. This means that a preamplification according to the invention using an enzyme mixture of Taq polymerase and a polymerase without proof-reading activity not only leads to an increased sensitivity of amplification reactions and microsatellite analyses, but also to amplification products that have no errors in their sequence and can therefore be used for mutation analyses.

Example 7

Analysis of disseminated tumour cells by RFLP using Ki-ras as an example

10 ml punctured bone marrow aspirate from pancreatic tumour patients or colon carcinoma patients was purified by means of a Ficoll density gradient. A total of 2 x 10^6 bone marrow cells were immunohistochemically stained with an anti-CK18 antibody (CK2 clone, Boehringer Mannheim) according to the manufacturer's instructions. CK18-positive cells were microdissected from the cytospots as single cells or in clusters with very low cell counts using a laser microdissector (Schütze, 1994). They were then lysed enzymatically with proteinase K according to the invention similarly to example 1 and a preamplification was carried out using a mixture of Taq polymerase and Pwo polymerase (10:1 mixing ratio).

The restriction fragment length polymorphism was analysed according to Trümper's protocol by enrichment PCR (Trümper et al., 1994). For this a 157 bp

fragment of the Ki-ras gene was amplified with 1 U Expand HiFi polymerase from a tenth of the batch of a preamplification PCR according to the invention containing 0.5 μM each of the primers 5'BstNI (Seq. ID. No. 13) and 3'WT (Seq. ID. No. 14) in a volume of 50 μl in 16 thermal cycles at an annealing temperature of 57°C. Subsequently a 16 μl aliquot was digested with 20 U MvaI (BstNI Isoschizomer) in a volume of 20 μl. 20 μl Mval-digested fragment and 10 μl undigested fragment were amplified in a second PCR using 0.5 μM each of 5'BstNI primer and 3'BstNI primer (Seq. ID. No. 15) in a total volume of 50 μl during 35 thermal cycles at an annealing temperature of 60°C. Afterwards 30 μl of the mixture was digested with the MvaI and, after adding 7 μl non-denaturing sample buffer, was analysed by gel electrophoresis on a non-denaturing 10 % polyacrylamide gel.

As shown in figure 7 it was possible to successfully amplify DNA from all samples. The existence of DNA fragments with a size of 143 bp after MvaI digestion is evidence of the mutation in all the investigated tumour material (lanes 1-7). In contrast all control reactions carried out with DNA from cells that cannot be stained with anti-CK18 (lanes 8-11) result in a non-mutated 114 bp product. Hence a method has been developed for the first time that can be used to unequivocally diagnose several mutations at a high rate of detection from a separated, disseminated tumour cell.

Example 8

Preamplification of reverse-transcribed RNA

Colorectal carcinoma cells of the cell line Lovo (ATCC) were separated with the aid of a fluorescence-activated cell sorter. Poly(A) RNA was isolated from the individual cells with the aid of the Dynabead mRNA Direct Kit (Dynal, Hamburg). The entire mixture was reverse transcribed with the aid of SuperScript RNaseH$^-$ reverse transcriptase (Life Technologies) using oligo(dT) primers (20 μl according to the manufacturer). The resulting cDNA (20 μl) was used as a template for an amplification according to the invention in accordance with the preamplification PCR protocol described in example 1. 1/30 of the cDNA preamplified in this manner was subsequently used as a template to amplify a specific 408 bp fragment

of the β2-microglobulin gene. 1/10 aliquots of the cDNA were used for amplification in a corresponding control reaction. The PCR was carried out under standard conditions at an annealing temperature of 60°C, 50 cycles and in the presence of 0.2 mmol dNTP, 5 µmol PCR primer (sequence ID No. 16 and 17) and 0.54 units Expand HiFi polymerase in a volume of 30 µl. As shown in figure 7 an amplification product was obtained from preamplified cDNA in 9 out of 12 analysed single cells whereas the use of DNA that was not preamplified according to the invention did not result in any detectable amplification products.

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SEQUENCE PROTOCOL

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Boehringer Mannheim GmbH
(B) ROAD: Sandhofer Strasse 116
(C) CITY: Mannheim
(E) COUNTRY: GER
(F) ZIP CODE: 68305
(G) TELEPHONE: 06217591456
(H) FAX: 06217594457

(ii) TITLE OF INVENTION: Improved method of primer extension preamplification PCR

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM.
(A) DATA CARRIER: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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22

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(A) LENGTH: 20 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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20

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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(2) INFORMATION FOR SEQ ID NO: 5:

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(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

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(ii) TYPE OF MOLECULE: genomic DNA

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(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
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(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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16

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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16

(2) INFORMATION FOR SEQ ID NO: 10:

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(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

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(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

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(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
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(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

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(2) INFORMATION FOR SEQ ID NO: 13:

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(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

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30

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(A) LENGTH: 20 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

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(2) INFORMATION FOR SEQ ID NO: 15:

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(A) LENGTH: 20 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

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20

(2) INFORMATION FOR SEQ ID NO: 16:

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(A) LENGTH: 21 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

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(2) INFORMATION FOR SEQ ID NO: 17:

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(A) LENGTH: 23 base pairs
(B) TYPE: nucleotide
(C) STRANDEDNESS: single strand
(D) TOPOLOGY: linear

(ii) TYPE OF MOLECULE: genomic DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

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23

Claims

1. Method for the amplification of nucleic acid fragments from a sample which comprises two or three thermocyclic amplification reactions wherein completely randomized primers are used during the first amplification reaction and specific primers are used in the second amplification reaction, characterized in that
a mixture of at least two DNA polymerases is used to amplify the DNA of which at least one polymerase has 3' – 5' exonuclease activity.

2. Method as claimed in claim 1, characterized in that
two or more elongation steps at different temperatures are carried out in the majority of cycles during the first thermocyclic amplification reaction.

3. Method as claimed in claim 1-2, characterized in that
the sample is obtained by cell lysis with the aid of an enzymatic protease digestion.

4. Method as claimed in claim 1-2, characterized in that
the sample consists of a pool of cDNAs.

5. Method as claimed in claim 1-4, characterized in that
the nucleic acid used is obtained from tissue sections.

6. Use of DNA which has been amplified as claimed in claims 1-5 for mutation analysis.

7. Use of DNA which has been amplified as claimed in claims 1-5 as a template for sequencing reactions.

8. Use of DNA which has been amplified as claimed in claims 1-5 for analysing microsatellites.

9. Method for the amplification of nucleic acid fragments from a sample which comprises two or three thermocyclic amplification reactions wherein completely randomized primers are used during the first amplification reaction and specific primers are used in the second amplification reaction and the sample contains a quantity of nucleic acid corresponding to an equivalent of no more than 100 cells, characterized in that
the amplificates are formed with a probability of more than 90 %.

10. Method as claimed in claim 9, wherein a quantity of nucleic acid is used as the sample which corresponds to an equivalent of no more than 10 cells, characterized in that
the amplificates are formed with a probability of more than 90 %.

11. Method as claimed in claim 9, wherein a quantity of nucleic acid is used as the sample which corresponds to an equivalent of no more than 5 cells, characterized in that
the amplificates are formed with a probability of more than 90 %.

12. Method as claimed in claim 9, wherein a quantity of nucleic acid is used as the sample which corresponds to an equivalent of one cell, characterized in that
the amplificates are formed with a probability of more than 50 %.

13. Method for the analysis of microsatellites which comprises two or three thermocyclic amplification reactions wherein completely randomized primers are used during the first amplification reaction and specific primers are used in the second amplification reaction and the sample contains a quantity of nucleic acid corresponding to an equivalent of no more than 100 cells, characterized in that

the amplificates of both alleles are formed with a probability of more than 90 %.

14. Method as claimed in claim 13, wherein a quantity of nucleic acid is used as the sample which corresponds to an equivalent of no more than 10 cells, characterized in that the amplificates are formed with a probability of more than 90 %.

15. Method as claimed in claim 13, wherein a quantity of nucleic acid is used as the sample which corresponds to an equivalent of no more than 5 cells, characterized in that the amplificates of both alleles are formed with a probability of more than 50 %.

Abstract

The invention concerns a method for the amplification of nucleic acid fragments from a sample which comprises two or three thermocyclic amplification reactions wherein completely randomized primers are used during the first amplification reaction and specific primers are used in the second amplification reaction which is characterized in that a mixture of at least two DNA polymerases is used to amplify the DNA of which at least one polymerase has proof-reading activity. In this manner it is possible to use DNA from individual cells or cell clones with low cell count for mutation analysis.

Figure 1 of 8

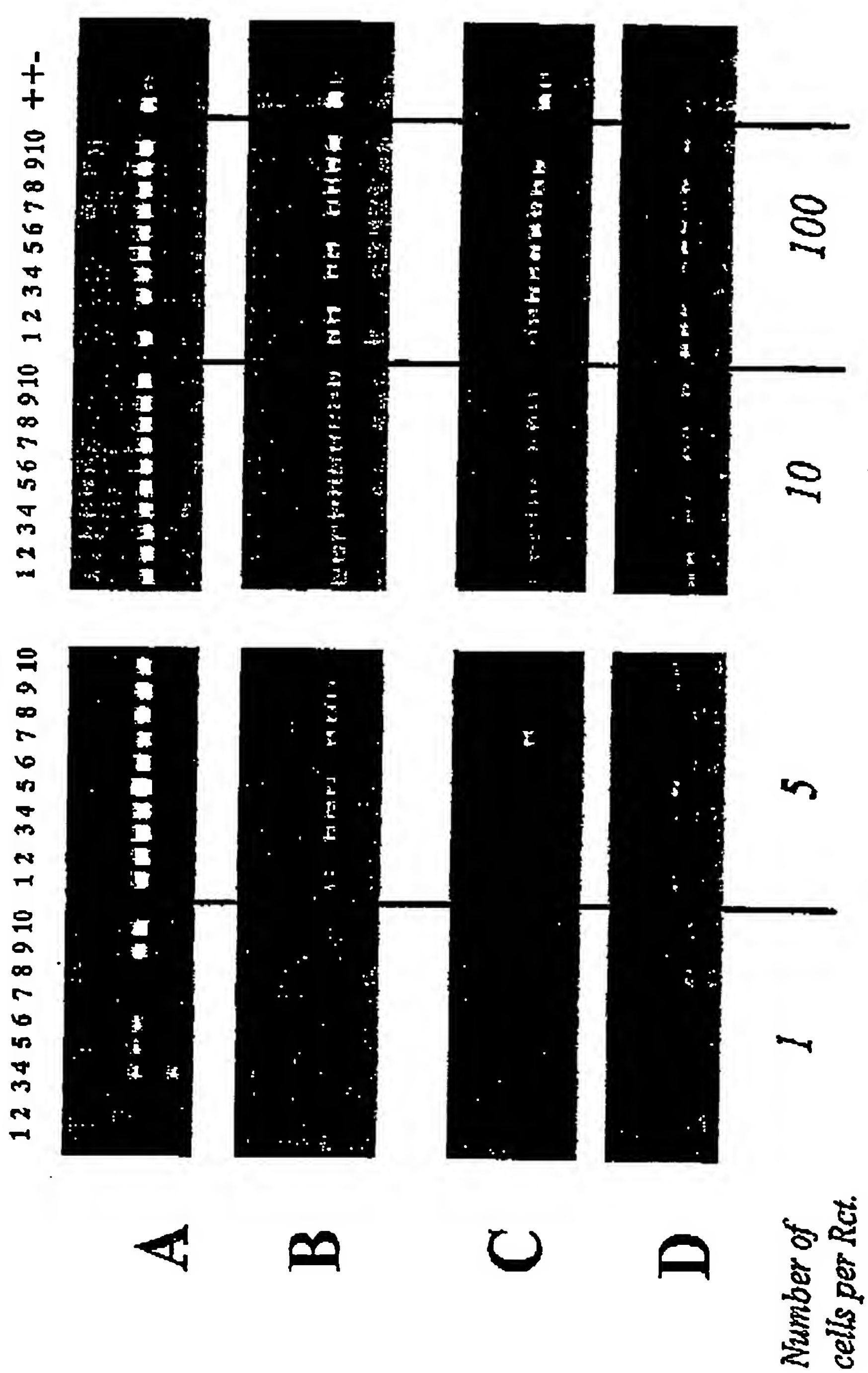


Fig.1

BEST AVAILABLE COPY

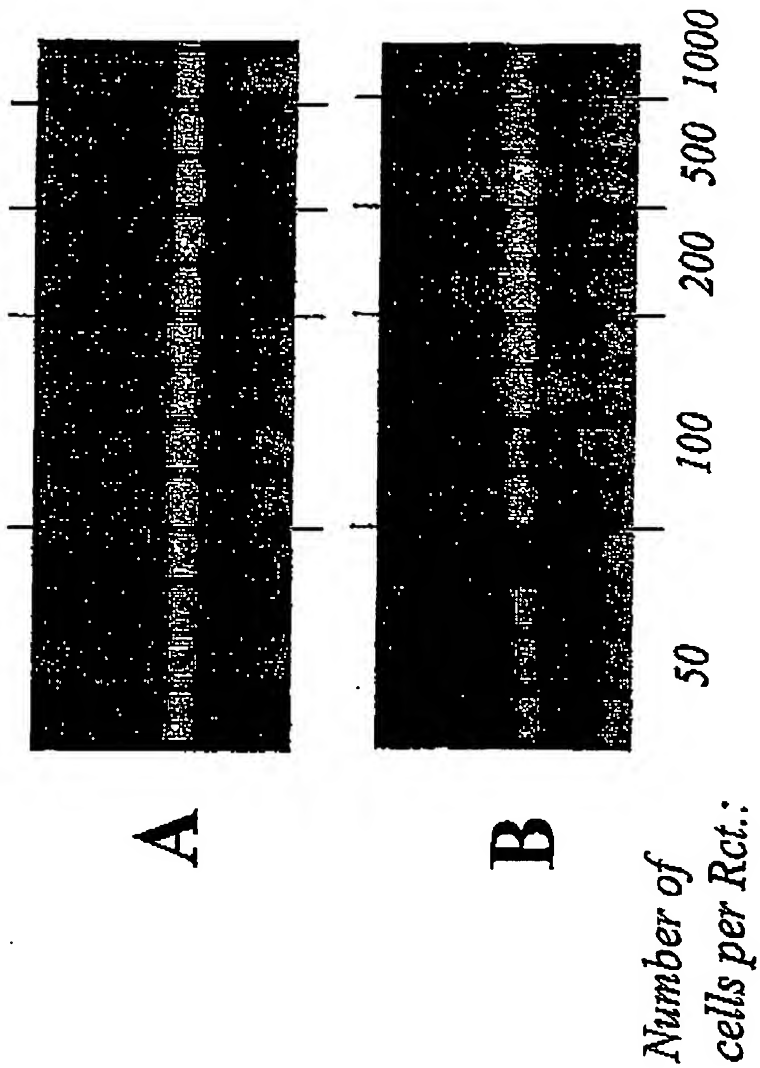
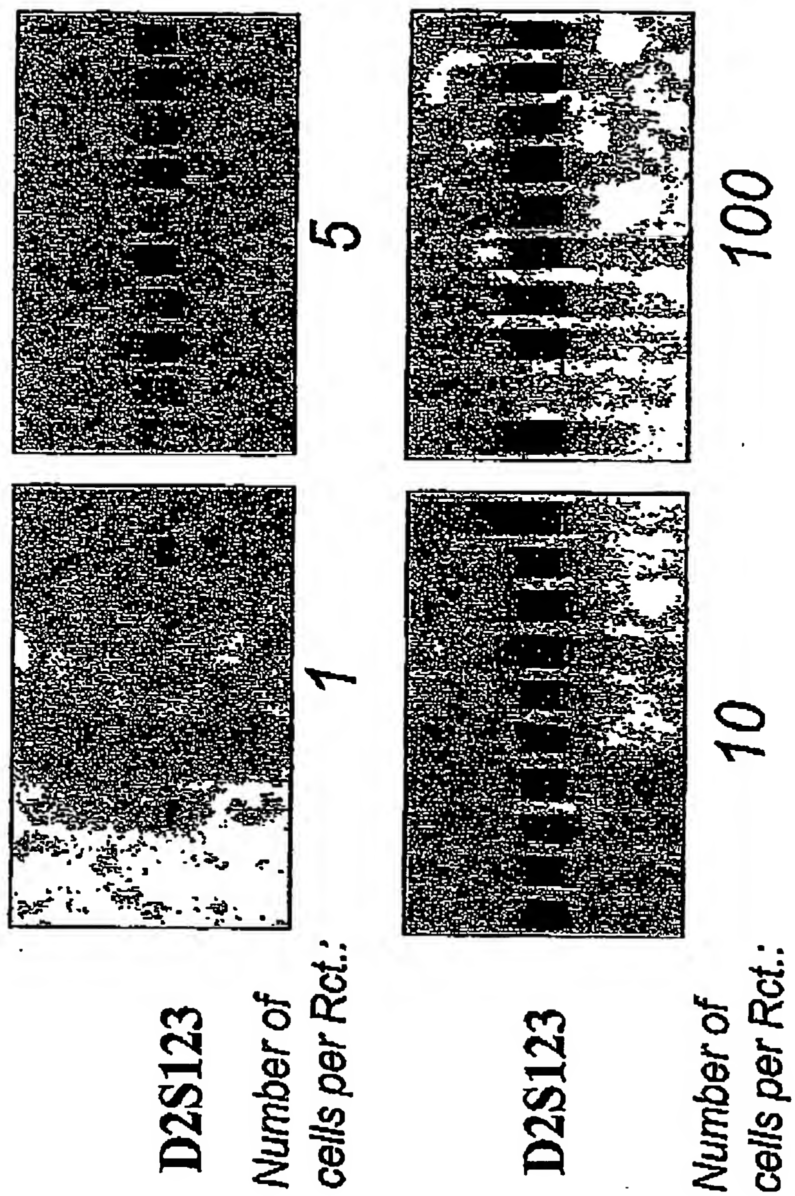


Fig. 2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Number of cells per Rct.:
1
5
D2S123
Number of cells per Rct.:
10
100

Fig. 3

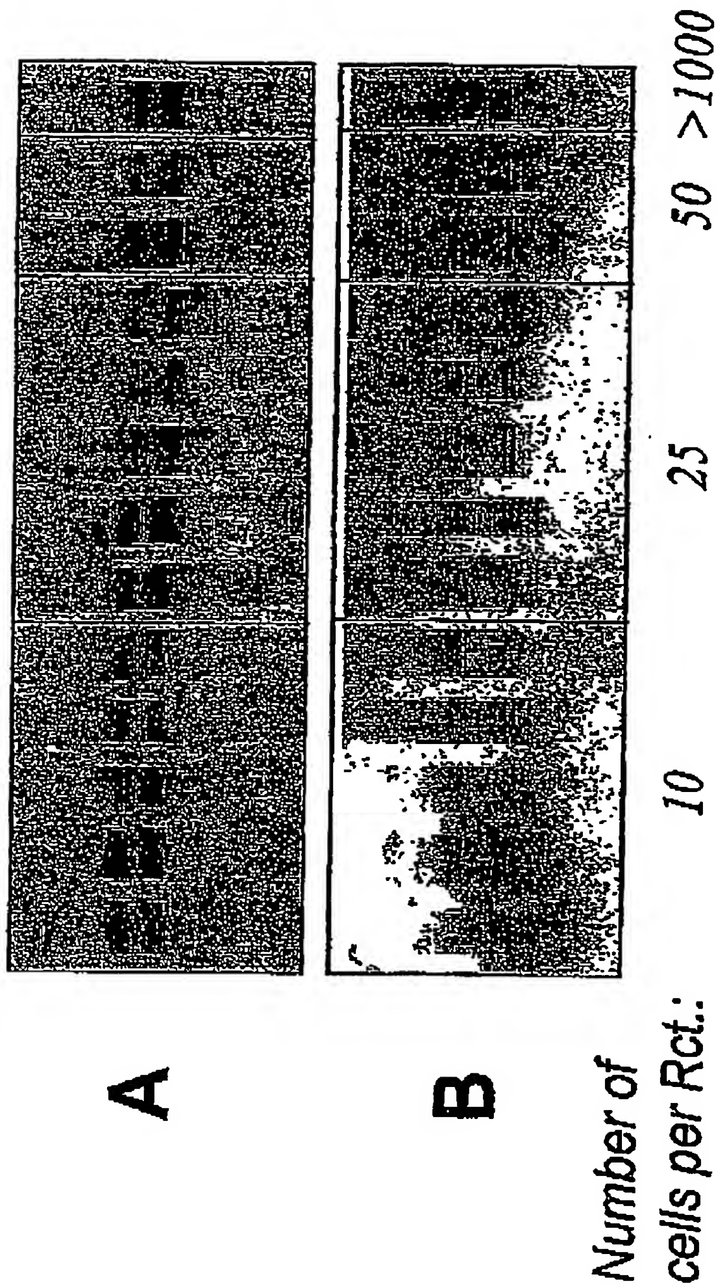

Fig. 4

Figure 5 of 8

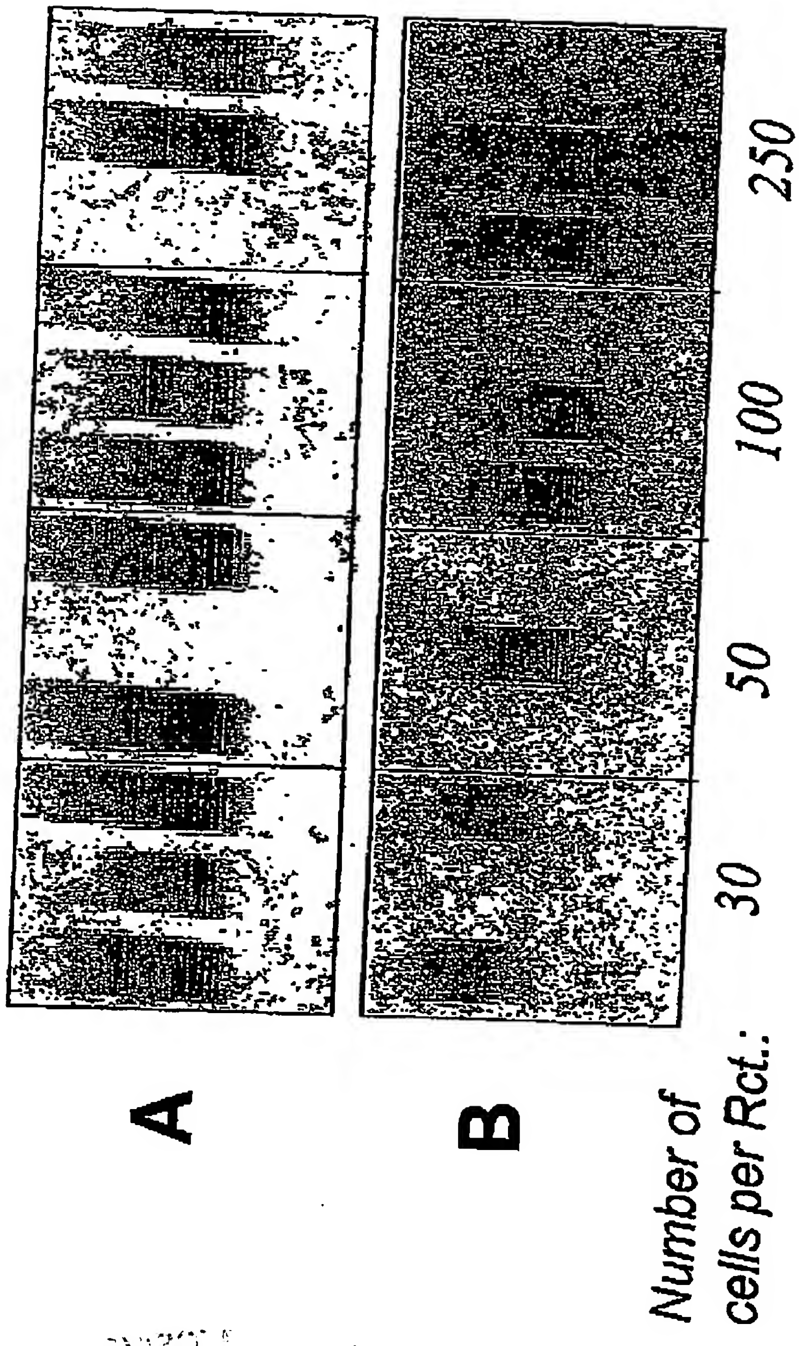

Fig. 5

Figure 6 of 8

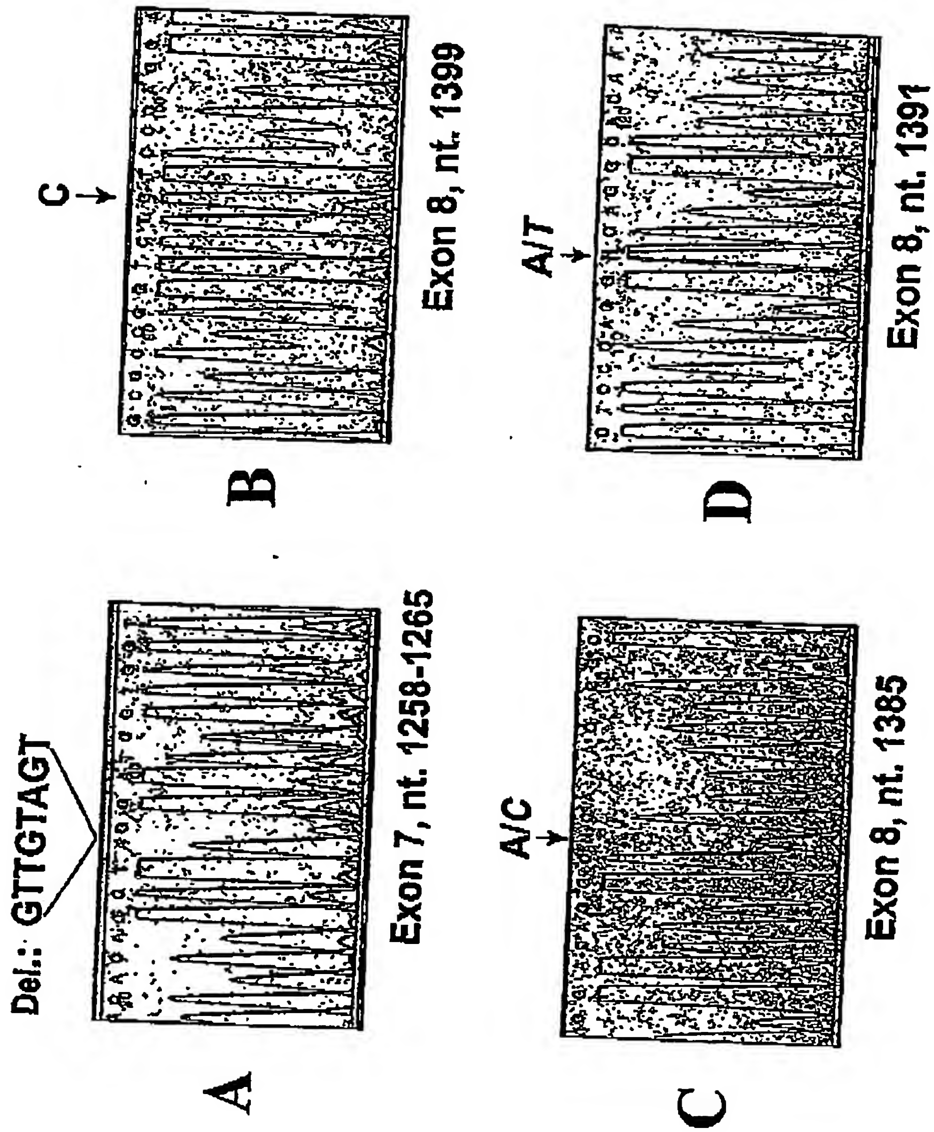

Fig.6

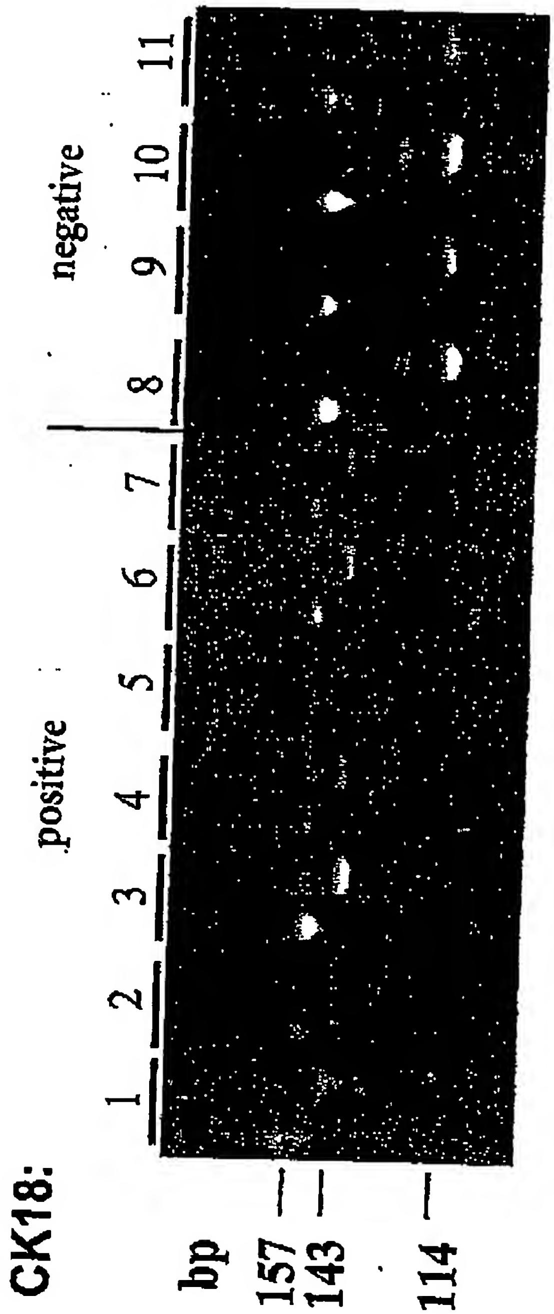

Fig. 7

Figure 8 of 8

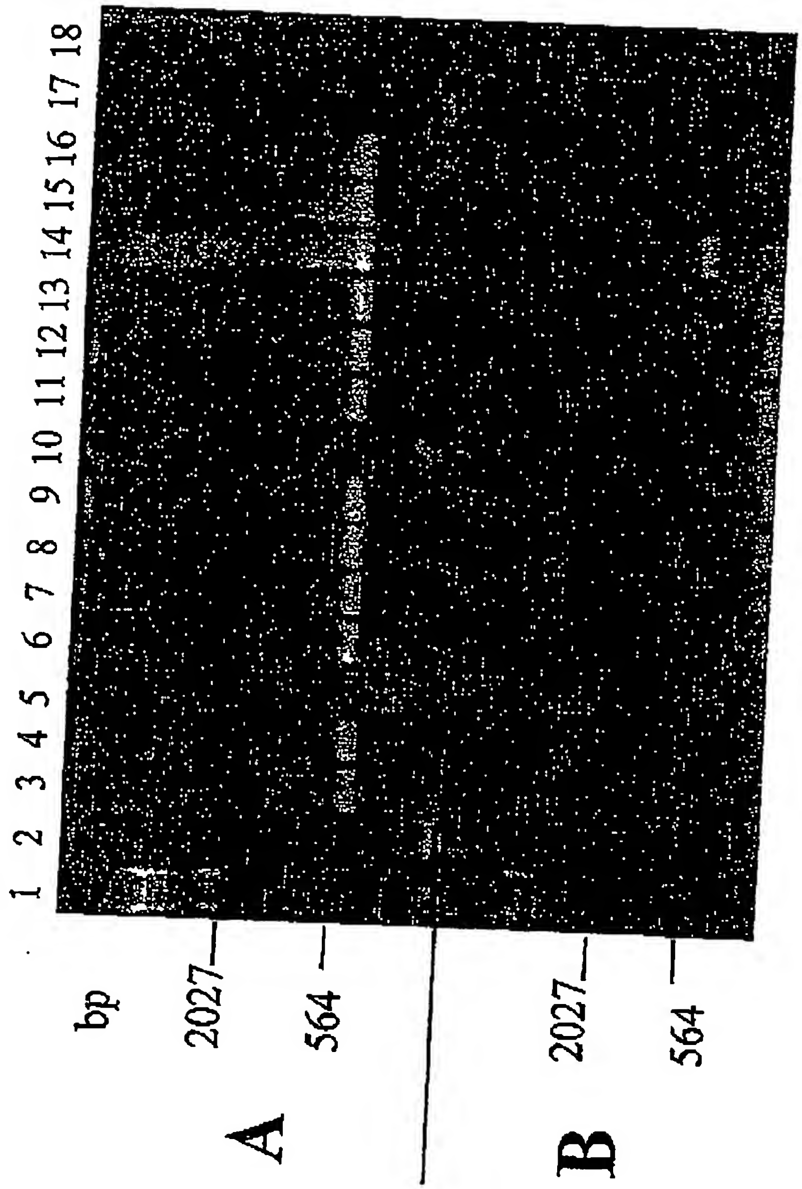

Fig.8